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Short communication

# Maternal separation induces anhedonia in female heterozygous serotonin transporter knockout rats

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ABSTRACT

The serotonin transporter (SERT) gene has been linked to depression, especially the short allele of the serotonin transporter linked polymorphic region (5-HTTLPR). When short allele carriers are exposed to stressful life events, their risk for developing depression is increased. The neurochemical properties of the short allele of the 5-HTTLPR in humans can be mimicked in heterozygous serotonin transporter knockout ($SERT^{+/-}$) rats. These animals have a similar reduction in SERT expression as humans with a 5-HTTLPR short allele. Several stress protocols have been used in $SERT^{+/-}$ animals but behavioural outcomes were mixed. Many studies used males to examine the behavioural effects of stress in $SERT^{+/-}$ rats, ignoring possible effects in females. However, women are depressed twice as often compared to men, therefore it is of great importance to study the effects of stress in females as well. Because early postnatal adversity can contribute to the psychopathology of depression, especially in vulnerable individuals, our aim was to investigate the effects of early-life stress in female $SERT^{+/-}$ rats and determine whether female $SERT^{+/-}$ rats could model the human short allele 5HTTLPR carriers.

To this end, $SERT^{+/-}$ rats were maternally separated for six hours a day from postnatal day 2-15. Control rats were handled for 15 min from PND2-15 to control for litter disturbances. In adulthood, female rats were assessed for affective, social and coping behaviour. In addition, nerve growth factor (NGF) gene expression in the basolateral amygdala (BLA) and paraventricular nucleus of the hypothalamus (PVN) and basal plasma corticosterone levels were measured.

Results show that maternal separation lowered sucrose preference in female $SERT^{+/-}$ rats compared to control $SERT^{+/-}$ rats, reflecting anhedonic behaviour. In addition, compared to control $SERT^{+/-}$ rats, maternal separation significantly lowered NGF gene expression in $SERT^{+/-}$ rats in both BLA and PVN, but did not affect plasma corticosterone levels. Together, these results show that early-life stress in female $SERT^{+/-}$ rats leads to depression-like behaviour and related plasticity impairments in the BLA and PVN.

The interaction of the serotonin transporter (SERT) and stressful life events (SLE) and its role in the etiology of depression throughout development and into adulthood has generated much interest since Caspi et al. showed this link [1]. People carrying the short allele for a polymorphism in the SERT linked polymorphic region appear to be at increased risk to develop depression when they are exposed to SLE, while people carrying the long allele appear more resilient. Multiple replication studies have been performed after this pioneering study, but with mixed results. A recent meta-analysis concluded that if SLE increases the risk of developing depression in people carrying the short-allele, this must be of modest effect size and only observable in limited situations [2].

Animal studies in SERT knockout rodents have been performed to elucidate the underlying mechanisms of SERT x SLE interactions. Heterozygous SERT knockout animals ($SERT^{+/-}$) are neurochemically comparable to the human S-allele carrier [see: 3,4] and may therefore be of translational value. However, most studies in $SERT^{+/-}$ rodents fail to show an interaction effect of SERT genotype and early-life stress on depression-like behaviour [see: 3]. The question arises whether the stressors used in these animal studies were severe enough to induce the depression-like phenotype needed to study the related underlying molecular mechanisms. In the current study, we applied 6-hours of maternal separation from postnatal day (PND)2-15 as an early SLE to $SERT^{+/-}$ rats. This is substantially longer compared to the studies described thus far. Since almost all rodent studies have been performed solely in males [3], it is unclear how female $SERT^{+/-}$ rodents respond to

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SLE. The use of females is of great importance since depression is more prevalent in women than in men [5]. We therefore studied the early-life stress effects in female SERT$^{+/-}$ rats. In the present study, we only assessed the effects of maternal separation in SERT$^{+/-}$ rats compared to control handled SERT$^{+/-}$ rats. It was our aim to develop a translational model in which stress induces a depression-like phenotype in heterozygous SERT deficient females, therefore we did not take other SERT genotypes into account. An early-life stress protocol was chosen as it has been shown that separating pups from the dam is a highly potent stressor activating the hypothalamus-pituitary-adrenal (HPA) [6]. The long-term effects of maternal separation are generally greater when the separation occurs early in life and last for longer periods [reviewed in: 7]. We performed several behavioural tests to study potential alterations in affective, social and coping behaviour due to early-life stress.

Moreover, we assessed neuronal plasticity in the brain, because this process is essential in order to adapt to a stressful environment, and because neurotrophins play a crucial role in plasticity during development [8,9]. The neurotrophin 'nerve growth factor' (NGF) plays a role in the pathophysiology of depression as NGF levels were found to be reduced in brains of depressed suicide victims [8]. In the current study, we assessed NGF gene expression levels in the basolateral amygdala (BLA) and paraventricular nucleus of the hypothalamus (PVN), two brain areas that are activated by stress [10]. Glucocorticoids are important in the stress-response as they act directly on the central nervous system and influence behaviour. Glucocorticoid levels, and especially cortisol (corticosterone in rodents), are often higher in major depression [11]. We therefore characterized basal plasma corticosterone (CORT) concentrations in maternally separated (MS) and control (CTR-) SERT$^{+/-}$ rats. We hypothesized that MS-SERT$^{+/-}$ rats would (1) display altered affective behaviour, (2) exhibit lower NGF gene expression, and (3) have higher basal plasma CORT levels compared to CTR-SERT$^{+/-}$ rats.

To test our hypothesis, we performed the following maternal separation protocol. Wistar SERT$^{+/-}$ rats (Slc6a41Hubr) were bred by crossing SERT$^{+/-}$ females with SERT$^{+/-}$ males. Dams were checked daily for delivery of pups, which was set as PND0. On PND2-15, pups were maternally separated as a whole litter for 6 h a day. During MS, whole litters were placed in preheated Makrolon type 2 cages (PND2-8: 32 ± 1$^0$C; PND9-15: 28 ± 1$^0$C). CTR pups were handled on PND2-15 for 15 min to control for litter disturbances. At PND21, pups were weaned and ears were punched for identification and genotyping (for genotyping protocol see: [12]). Pups were socially housed with same-sex animals. Only female SERT$^{+/-}$ rats were used in this study

We used two batches of adult animals; batch 1 to assess behaviour and CORT levels and batch 2 to measure NGF gene expression levels in the BLA and PVN. For batch 1, 24 CTR animals were used coming from 11 litters and 15 MS animals were used originating from 7 litters. For batch 2, 8 CTR animals were used from 7 litters and 7 MS animals were used originating from 7 litters. The animals in batch 1 were tested between the ages of 10–16 weeks. Tests were at least one week apart in the following order: open field (OF), sociability and social recognition, elevated plus maze (EPM), sucrose preference, CORT levels, and forced swim test (FST). Animals in batch 2 were sacrificed at the age of six months. Animals were socially housed under standard laboratory conditions with a reversed 12h:12 h light/dark cycle (lights off at 11:00 a.m.) with *ad libitum* access to food (RMH-B, AB Diets; Woerden, the Netherlands) and tap water, unless stated otherwise. By measuring vaginal wall impedance (model MK-11, Muromachi, Tokyo, Japan) it was determined whether females were in oestrus before testing. Females were only tested when not in oestrus, unless stated otherwise. Behavioural testing took place between 12:00 and 17:00 p.m. All experimental procedures were approved by the Groningen University Committee of Animal experiments.

Animals (batch 1) were individually housed and habituated with two water bottles, one on each side of the cage, for 3 consecutive days. Following habituation, animals were presented with one water bottle and one bottle containing a sucrose solution for 24 h on alternating days. On the other days two bottles of water were presented. With each sucrose day, the sucrose concentration increased with 0.25% (0.25% to 1%). Sucrose bottle locations on the cage were alternated on sucrose days to prevent spatial bias. Fluid consumption (gram) was determined daily. The preference for sucrose above water was calculated ((sucrose intake (g)/total intake (g)) x 100%). Sucrose intake was corrected for body weight. For gene expression analysis (batch 2) six-months-old females were decapitated, brains were collected, frozen in isopentane on dry ice and stored at −80 °C. Brains were cut into coronal slices (200 μM; −12 °C) and punches from the PVN and BLA were obtained according to the atlas of Paxinos and Watson. For further material and methods of RNA extraction, cDNA, qPCR, OF, social recognition, EPM, CORT, FST and data analysis methods: see supplementary section. Sucrose preference was analysed using a one-way ANOVA for repeated measures with treatment (CTR/MS) as between subject factor, and the sucrose concentrations as within subject variables. When a significant main or interaction effect was found, data were further analysed using an independent samples *t*-test for between subject effects at different sucrose concentrations. For all other tests (behaviour, NGF expression and CORT) an independent sample *t*-test was used. Outliers were excluded from the data, and level of significance was set at $p < 0.05$. All statistical analyses were performed using the Statistical Package for Social Sciences for Windows version 22.0 (SPSS, Chicago, IL, USA).

For the sucrose preference, an overall maternal treatment ($F_(1,38_) = 5.21$; $p < 0.05$) and sucrose concentration ($F_(3,114_) = 17.84$; $p < 0.001$) effect was found, with a higher preference for sucrose in control animals, while sucrose preference increased with increasing sucrose concentrations in both groups (Fig. 1). No interaction between maternal treatment and sucrose concentration was found ($F_(3,114_) = 1.21$; ns). Post hoc analysis revealed that compared to CTR-SERT$^{+/-}$ rats, a lower preference for sucrose was found for MS-SERT$^{+/-}$ rats at concentrations of 0.5% ($t_{(1.34)} = 2.45$, $p < 0.05$), 0.75% (tendency: $t_(1,36_) = 1.75$, $p = 0.09$) and 1.0% ($t_(1,35_( = 2.25$, $p < 0.05$).

NGF gene expression was downregulated in the BLA ($t_(1,14_) = 3.10$; $p < 0.01$) and PVN ($t(1,14_) = 2.42$; $p < 0.05$) of MS-SERT$^{+/-}$ relative to CTR-SERT$^{+/-}$ rats (Fig. 2).

Since no statistical differences were found for the OF, social recognition, EPM, CORT and FST these results can be found in the supplementary data.

In the present study, we investigated the effects of MS in female SERT$^{+/-}$ rats on affective behaviour, NGF brain expression and plasma CORT levels in adulthood. By applying MS for 6 h a day from PND2-15 in SERT$^{+/-}$ rats we first showed that MS-SERT$^{+/-}$ rats have lower sucrose preference compared to CTR-SERT$^{+/-}$ rats, suggesting that MS-SERT$^{+/-}$ rats display anhedonia-like behaviour relative to CTR-SERT$^{+/-}$ rats. Secondly, we showed that NGF gene expression is significantly lower in the BLA and PVN of MS-SERT$^{+/-}$ rats compared to CTR-SERT$^{+/-}$ rats, suggesting that neurotrophic plasticity is altered by

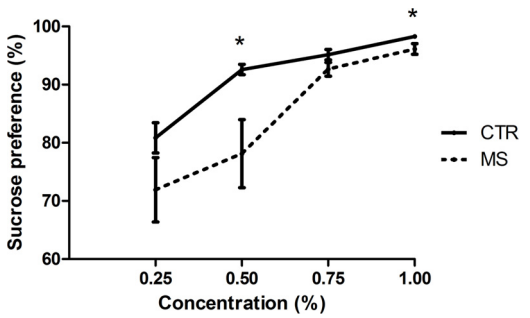


**Fig. 1.** Sucrose preference.
Sucrose preference (mean ± SEM) at different sucrose concentrations in control (CTR) and maternally separated (MS) female SERT$^{+/-}$ rats. * = $p < 0.05$.

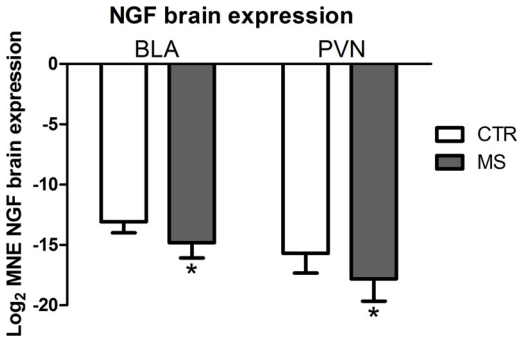


**Fig. 2.** Nerve growth factor gene expression in the brain.
Nerve growth factor (NGF) gene expression (mean ± SEM) in the basolateral amygdala (BLA) and paraventricular nucleus of the hypothalamus (PVN) in control (CTR) and maternally separated (MS) female SERT$^{+/-}$ rats, $\log_2$MNE = $\log_2$ Mean normalized expression. * = $p < 0.05$.

MS in female SERT$^{+/-}$ rats. Basal CORT levels were not altered by MS in SERT$^{+/-}$ rats. Together, these results indicate an anhedonic-like phenotype with related plasticity impairments in female SERT$^{+/-}$ rats exposed to stress early in life.

Anhedonia is characterized by a reduced sensitivity to natural rewards like weak sucrose solutions, which is present in animal models of early-life stress [13]. SERT$^{+/-}$ rats exposed to maternal separation also display anhedonia when using weak sucrose solutions compared to CTR-SERT$^{+/-}$ rats. Interestingly, sensitivity to natural rewards can be restored by several weeks of antidepressant treatment [14]. Whether this is the case in our model remains to be established. A mice study of Tang and colleagues [15] found that unpredictable chronic stress and SERT deficiency can induce anhedonia. Since our SERT$^{+/-}$ rats display reduced SERT protein expression, this might play a role in the anhedonia seen in SERT$^{+/-}$ rats as well. Even though we do not know whether the anhedonia levels of SERT$^{+/-}$ rats differ from SERT$^{+/+}$ rats, sucrose preference were lowered in MS-SERT$^{+/-}$ relative to CTRL-SERT$^{+/-}$ rats, showing that early-life stress in female SERT$^{+/-}$ rats leads to depression-like behaviour.

Despite the significant difference in sucrose preference, we did not find any significant differences in other behavioural tests. Although no effects were seen on thigmotaxis in the OF, MS-SERT$^{+/-}$ rats did tend to move less ($p = 0.08$) in the OF compared to controls. This trend in moving less on the OF is in line with a study in which SERT$^{+/-}$ mice that received low maternal care displayed enhanced anxiety-like behaviour in the OF (less time spent in the centre) compared to those that received high maternal care [16]. The latter authors also found a reduced latency to reach immobility in the tail suspension test, but this altered stress coping was not found in our MS-SERT$^{+/-}$ rats, possibly because we used different species, sex and a different behavioural test setup to assess stress coping. In contrast, another study with SERT$^{+/-}$ rats found improved stress coping behaviour in SERT$^{+/-}$ animals exposed to MS compared to controls [17]. However, this study used an escapable shock stress. In addition, a 3-hour MS protocol was used, indicating that the severity of the stressor (low stress improving stress coping) might play a role as well. In a mouse study With respect to female SERT$^{+/-}$ animals and the use of early-life stress, female SERT$^{+/-}$ mice exposed to prenatal stress tended to show decreased mobility in the FST [18]. In the same study, a decrease in anxiety-like behaviour in SERT$^{+/-}$ mice exposed to prenatal stress was found. Reasons for these discrepancies in anxiety-like behaviour might be due to the different stressors used. Nevertheless we found a substantially lowered sucrose preference in SERT$^{+/-}$ rats exposed to stress, suggesting the presence of a symptom of a depression-like phenotype, namely anhedonia.

On a social level, we found no differences in sociability and social recognition between CTR-SERT$^{+/-}$ and MS-SERT$^{+/-}$ rats. This contrasts with a study performed in SERT$^{+/-}$ mice where an increased social avoidance towards an unfamiliar male in a novel environment was found when experiencing high levels of stress [19]. However, mice used in this study were subjected to chronic psychosocial stress, which is different from the MS protocol we used and may contribute to the difference in sociability outcome.

Because faecal CORT levels were increased upon three-day resident-intruder stress in SERT$^{+/-}$ mice [20], we hypothesized higher plasma CORT levels in MS-SERT$^{+/-}$ compared to CTR-SERT$^{+/-}$ l rats. Interestingly, MS-SERT$^{+/-}$ rats tended to show lower basal CORT levels than CTR-SERT$^{+/-}$ rats. In another study with MS-SERT$^{+/-}$ rats (3 h a day from PND2-15), no differences were found in CORT levels [21]. In addition, basal CORT levels in prenatally stressed SERT$^{+/-}$ mice were lower compared to wildtype mice, especially in males [18]. Their and our findings contrast with a study in SERT$^{+/-}$ mice where increased CORT levels were found 24 h after a last psychosocial stressor [19]. In our study, basal CORT levels were measured 12 weeks after the MS protocol, after most of the behavioural tests had already occurred, probably reflecting basal CORT levels. The small non-significant decrease in CORT levels might indicate an adaptation in the stress system preparing the animal for a stressful environment. This is only speculative, and further experiments are warranted to reveal whether the response to an acute stressor is altered in MS-SERT$^{+/-}$ rats.

Alterations in the morphology of neural cells due to chronic stress are often associated with a deficiency of neurotrophic factors. A shortage of neurotrophic factors can contribute to the pathogenesis of depression [22]. Related to our study, a reduction of brain-derived neurotrophic factor (BDNF) was found in the ventral hippocampus of male SERT$^{+/-}$ rats exposed to 3-hour maternal separation stress compared to control SERT$^{+/-}$ rats. However, also an increase of BDNF was found in the dorsal hippocampus and dorsomedial prefrontal cortex after the maternal separation in SERT$^{+/-}$ rats, indicating that early-life stress affects BDNF expression but in a different anatomical manner [23]. Although BDNF levels are often reported in animal models, NGF is also important with respect to alterations in the neural cell morphology. Glucocorticoids are known to regulate NGF in the brain and stress may alter NGF breakdown in the hypothalamus [24]. Moreover, NGF blood levels are decreased in patients with psychiatric disorders [25]. In the present study we showed that NGF is significantly lowered in the basolateral amygdala and paraventricular nucleus of the hypothalamus of MS-SERT$^{+/-}$ rats compared to CTR-SERT$^{+/-}$ rats, indicating that adult NGF gene expression is altered by early postnatal MS in female SERT$^{+/-}$ rats. To prevent changes in gene expression as a result of the behavioural tests, NGF levels were measured in a different group of animals, and thus cannot be correlated to observed behavioural effects. Also, we cannot rule out whether the effects seen are due to maternal separation alone, or whether the SERT genotype also plays a role.

One limitation of the present study is that no comparisons were made with SERT$^{+/+}$ animals. Therefore we cannot speculate whether SERT$^{+/-}$ rats are more vulnerable to maternal separation when compared to SERT$^{+/+}$ rats. Moreover, we did not include males, therefore it remains to be established whether females SERT$^{+/-}$ rats are indeed more sensitive to this MS protocol than males. In conclusion, the present data indicate that MS in the early postnatal period induces anhedonia in female SERT$^{+/-}$ rats and that NGF might contribute to this observed effect. Whether outcomes in female SERT$^{+/-}$ rats are different from female SERT$^{+/+}$ or SERT$^{-/-}$ rats remains to be investigated.

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